*Am. J. Hum. Genet.* 62:562–572, 1998

# Biochemical Characterization of Arylsulfatase E and Functional Analysis of Mutations Found in Patients with X-Linked Chondrodysplasia Punctata

Aurora Daniele,[1,*,†] Giancarlo Parenti,[2,*] Marilena d'Addio,[2] Generoso Andria,[2] Andrea Ballabio,[1] and Germana Meroni[1]

[1]Telethon Institute of Genetics and Medicine, San Raffaele Biomedical Science Park, Milan; and [2]Department of Pediatrics, Federico II University, Naples

## Summary

**X-linked chondrodysplasia punctata (CDPX) is a congenital disorder characterized by abnormalities in cartilage and bone development. Mutations leading to amino acid substitutions were identified recently in CDPX patients, in the coding region of the arylsulfatase E (ARSE) gene, a novel member of the sulfatase gene family. Transfection of the ARSE full-length cDNA, in Cos7 cells, allowed us to establish that its protein product is a 60-kD precursor, which is subject to N-glycosylation, to give a mature 68-kD form that, unique among sulfatases, is localized to the Golgi apparatus. Five missense mutations found in CDPX patients were introduced into wild-type ARSE cDNA by site-directed mutagenesis. These mutants were transfected into Cos7 cells, and the arylsulfatase activity and biochemical properties were determined, to study the effect of these substitutions on the ARSE protein. One of the mutants behaves as the wild-type protein. All four of the other mutations resulted in a complete lack of arylsulfatase activity, although the substitutions do not appear to affect the stability and subcellular localization of the protein. The loss of activity due to these mutations confirms their involvement in the clinical phenotype and points to the importance of these residues in the correct folding of a catalytically active ARSE enzyme.**

Received June 9, 1997; accepted for publication January 6, 1998; electronically published February 25, 1998.

Address for correspondence and reprints: Dr. Germana Meroni, Telethon Institute of Genetics and Medicine, San Raffaele Biomedical Science Park, Via Olgettina 58, 20132 Milan, Italy. E-mail: meroni@tigem.it

* Drs. Daniele and Parenti contributed equally to this work.

† Present affiliation: Dipartimento di Biochimica e Biotecnologie Mediche, Medical School, Federico II University, Naples.



0002-9297/98/6203-0009$02.00

## Introduction

X-linked recessive chondrodysplasia punctata (CDPX; MIM 302950) is a congenital defect of bone and cartilage development, characterized by abnormal calcium deposits in regions of enchondral bone formation, severe underdevelopment of nasal cartilage, distal phalangeal hypoplasia, and short stature (Curry et al. 1984). Interestingly, CDPX displays phenotypic similarities to two disorders that are due to defects in vitamin K metabolism and function: Warfarin embryopathy (Hall et al. 1980) and vitamin K epoxide reductase deficiency (Pauli et al. 1987).

The CDPX critical region was mapped to the distal part of the short arm of the X chromosome, in Xp22.3 (Wang et al. 1995). In this region, we have identified a cluster of genes, those for arylsulfatase D (ARSD), arylsulfatase E (ARSE), and arylsulfatase F (ARSF) (Franco et al. 1995; Puca et al. 1997), that represent novel members of the sulfatase gene family. Mutation analysis of CDPX patients implicated ARSE as the gene responsible for the disease, and six mutations leading to amino acid substitutions were identified (Franco et al. 1995; Parenti et al., 1997).

Sulfatases are a group of enzymes that hydrolyze a sulfate ester bond in a variety of different compounds. In addition, a subset of these proteins, the arylsulfatases, are able to hydrolyze a sulfate group from artificial substrates containing a phenolic ring, such as 4-methylumbelliferyl (4MU) sulfate. Eleven human sulfatases have been identified and characterized thus far, and the importance of these enzymes is emphasized by the existence of inherited disorders that are due to specific sulfatase deficiencies (Ballabio and Shapiro 1995; Kolodny and Fluharty 1995; Neufeld and Muenzer 1995; Parenti et al. 1997). Moreover, a defect in a co- or posttranslational modification common to all known sulfatases is the basis of multiple sulfatase deficiency (MSD), an autosomal recessive disease in which all sulfatase activities are deficient (Kolodny and Fluharty 1995; Schmidt et al. 1995).

The genes for most sulfatases have been cloned by use

of functional approaches. The recently identified Xp22.3 sulfatases (ARSD, ARSE, and ARSF) were the only sulfatases isolated by a positional cloning effort that gave no indication of their substrate specificity and biochemical properties. Protein-sequence comparison revealed a high degree of homology with the arylsulfatases and particularly with steroid sulfatase (STS), the gene for which is located in the same chromosomal band, Xp22.3 (Ballabio et al. 1987; Yen et al. 1987). Genomic studies of human and primate X and Y chromosomes have indicated that the sulfatase genes present in Xp22.3 evolved from a common ancestor, thus representing a subfamily of arylsulfatases (Meroni et al. 1996). Biochemical studies, after transfection of ARSE cDNA in Cos7 cells, confirmed that the ARSE gene product is a heat-labile arylsulfatase enzyme that hydrolyzes 4MU sulfate at neutral pH (Franco et al. 1995). Although the natural substrate is still unknown, this arylsulfatase activity is competitively inhibited by Warfarin, suggesting the possible involvement of this enzyme in vitamin K metabolism (Franco et al. 1995; G. Parenti, unpublished data). In this article, we report the initial functional characterization of ARSE protein and the analysis of the effects of the mutations found in CDPX patients on the activity, stability, and subcellular distribution of this enzyme.

## Material and Methods

### *Site-Directed Mutagenesis and Plasmids*

Site-directed mutagenesis of the ARSE expression plasmid (pcDL-ARSE) (Franco et al. 1995) was performed by use of the commercially available Transformer II kit (Clontech), in accordance with the manufacturer's instructions. To introduce mutations R12S, G137V, R111P, and G245R into wild-type ARSE cDNA, the following oligonucleotides were used: 5′-GTT TGT GTT TCA GCA GCT GGC TGC (nt 89–112 of ARSE cDNA); 5′-CTG AAA GAG AAA GTC TAT GCC ACTG (nt 462–486 of ARSE cDNA); 5′-GCA TTG GTT ACC CTG TTC TTC AGT GG (nt 386–411 of ARSE cDNA); 5′-CTC CTA TTT TGT GCG TGC TCT GAT TG) (nt 686–711 of ARSE cDNA); and selection primer 5′-CAT CGA TAC GCG TCT GTG GAA TG (nt 2598–2621 of pcDL; Takebe et al. 1988). The mutation corresponding to C492Y was introduced into ARSE wild-type cDNA by two successive rounds of PCR using the following oligonucleotides: Tyr, 5′-GCC GGT GCC TAC TAT GGA AGA AAGG (nt 1531–1555 of ARSE cDNA), and TyrRev, 5′-CTT CCA TAG TAG GCA CCG GC (nt 1550–1531 of ARSE cDNA); MID3, 5′-CAG CTT TTC ACT GCA GAC ATT TAT TG (nt 2067–2092 of the cDNA); MID4, 5′-GCA TTA TTG TGA GAG GTT TCT GCA CG (nt 1664–1690 of the cDNA); and ASE16, 5′-GAC GTG TTC CCC ACC GTG GTC (nt 1540–1560 of the cDNA). In the preceding lists, the nucleotide substitutions are underlined. The DNA sequence of each mutant construct was confirmed by sequence analysis; for C492Y, the entire PCR product was sequenced. The ARSD expression vector was constructed by cloning of the full-length ARSD cDNA (Franco et al. 1995) into the pcDL vector (Takebe et al. 1988).

### *Cos7 Cell Transfection and Protein Extract*

Cos7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 g penicillin/ml, and 100 g streptomycin/ml, at 37°C in 5% $CO_2$ atmosphere, and 10 $\mu$g wild-type (ARSE or ARSD) and mutant (ARSE) cDNA expression vector constructs were introduced into Cos7 cells, by electroporation using a BioRad Gene Pulser apparatus, and were seeded in 25-$cm^2$ flasks. The cells were harvested 24–72 h after transfection and were resuspended in extraction buffer (150 mM NaCl; 100 mM Tris-HCl, pH 7.5; and 1% Triton X-100), and cell lysates were clarified by centrifugation at 13,000 rpm to remove cell debris. To isolate glycoproteins, cell extracts were diluted 1:10 in concanavalin A buffer (0.1 M acetate buffer, pH 6; 1 M NaCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; and 1 mM $MnCl_2$) and were incubated overnight with concanavalin A-Sepharose 4B (Sigma), at 4°C. Concanavalin A-Sepharose pellets were resuspended in SDS-PAGE sample buffer, boiled, and loaded on the gel. To determine the association of ARSE with membranes, cells either were lysed by sonication, in the presence of 0.5 M $Na_2CO_3$ (Fujiki et al. 1982), or were extracted with Triton X-114 (150 mM NaCl; 100 mM Tris-HCl, pH 7.5; and 1% Triton X-114), and the aqueous and detergent phases were separated as described elsewhere (Bordier 1981). As a control for transfection efficiency, 1 $\mu$g of a construct carrying a luciferase reporter gene under the control of a strong promoter cytomegalovirus was cotransfected, and luciferase activity was tested as described elsewhere (Nordeen 1988).

### *Arylsulfatase Activity*

Arylsulfatase activity, with the fluorogenic substrate 4MU sulfate, was assayed as described elsewhere (Franco et al. 1995).

### *Production of Polyclonal Antibodies*

The regions of amino acids 276–558 of ARSE and amino acids 390–593 of ARSD were fused to the six-histidine tag bacterial expression vector pQE (Qiagen) and were produced in *Escherichia coli,* after induction with isopropyl-$\beta$-D-thiogalactoside (IPTG). The His-tagged proteins were purified on an NiNTA agarose column (Qiagen) and were used to immunize rabbits. ARSE and ARSD antisera were precipitated with ammonium

sulfate, and the antibodies were purified on a protein A-Sepharose column.

### *Immunoblot*

Ten micrograms of soluble cellular proteins was boiled for 5 min in sample buffer, electrophoresed through 10% SDS-PAGE, and electroblotted onto nitrocellulose membranes (BioRad). The membranes were treated with 5% dry milk in Tris-buffer saline with the addition of Tween 20 (TTBS [20 mM Tris-HCl, pH7; 50 mM NaCl; and 0.1% Tween 20]), to inhibit nonspecific binding. Anti-ARSE antibody was used at a 1:200 dilution in TTBS. Visualization of antibody binding was performed with a secondary goat anti-rabbit IgG antibody conjugated with alkaline phosphatase.

### *Immunofluorescence*

Indirect immunofluorescence was performed on paraformaldehyde (PFA)-fixed transfected Cos7 cells. Cells were permeabilized with 0.2% Triton X-100, blocked with porcine serum, and incubated with anti-ARSE or anti-ARSD antibody (1:400). Staining was obtained after incubation with secondary fluorescein isothiocyanate (FITC)– or tetramethylrhodamine isothiocyanate (TRITC)-conjugated isotype-specific antibodies. Specific Golgi staining was obtained either by incubation of PFA-fixed cells with 15 $\mu$g rhodamine-conjugated wheat germ agglutinin (WGA)/ml (Sigma) or by double staining with two commercially available anti-Golgi monoclonal antibodies that recognize the $\beta$-COP (clone maD; Sigma) and the Golgi 58K (clone 58K-9; Sigma) proteins.

### *Biosynthetic Labeling and SDS Analysis*

Seventy-two hours after transfection, Cos7 cells were starved for 2 h with methionine-free DMEM (Sigma). The cells were labeled for 5–30 min with 150 mCi $^{35}$S-methionine, in methionine-free DMEM containing 2% dialyzed fetal bovine serum (FBS), and were chased for 1, 4, 8, and 20 h in DMEM medium supplemented with 10% FBS. The cells were harvested by scraping and were lysed in 100 $\mu$l 0.05 M Tris-HCl buffer, pH 7.5, containing 0.5% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl flouride (PMSF), and 1 mM EDTA. Cell lysates were clarified by centrifugation at 13,000 rpm, were diluted to 600 $\mu$l with 0.05 M Tris-HCl buffer containing 150 mM NaCl, 1 mM PMSF, and 1 mM EDTA, and were incubated for 2 h with 4 $\mu$l rabbit preimmune serum and 35 $\mu$l protein A-Sepharose suspension (1:1 in $H_2O$). After centrifugation at 1,500 rpm, ARSE was immunoprecipitated with 4 $\mu$l rabbit anti-ARSE antiserum for 4 h. Immune complexes were precipitated with 35 $\mu$l protein A-Sepharose suspension, were washed six times with 0.05 M Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM PMSF, and 1 mM EDTA, and were analyzed by SDS-PAGE, followed by fluorography.

### *Endoglycosidase H Treatment*

Transfected Cos7 cells were pulse labeled for 30 min, chased for 4 h, and treated as described above. Immune complexes were solubilized in 1.25 M Tris-HCl buffer, pH 6.8, containing 1% SDS, at 100°C for 10 min. Supernatants were diluted in 500 $\mu$l 0.1 M sodium acetate buffer, pH 6.0, containing 1% 2-mercaptoethanol and were incubated with 40 mU endoglycosidase H (Boheringer) for 6 h. ARSE polypeptides were precipitated with 10% tricholoroacetic acid (TCA) and were analyzed by SDS-PAGE.

## Results

### *Characterization of ARSE in Transfected Cos7 Cells*

We raised polyclonal antibodies against the predicted C-terminal portion of the ARSE and ARSD proteins. The regions of amino acids 276–558 of ARSE and amino acids 390–593 of ARSD were fused to a six-histidine tag bacterial expression vector and then were produced in *E. coli* and used to immunize rabbits. Although the antisera were raised against a region where these two sulfatases share the lowest degree of homology (70% identity) (Franco et al. 1995), both polyclonal antibodies crossreacted with all members of the subfamily of Xp22.3 sulfatases (ARSD, ARSE, ARSF, and STS), after transfection in Cos7 cells (data not shown), hampering the biochemical characterization of the endogenous ARSE product in cellular extracts. Therefore, we limited our study to the ARSE protein transiently expressed in Cos7 cells.

After 30 min of metabolic labeling of Cos7 cells transfected with the wild-type ARSE expression vector (Franco et al. 1995), two polypeptides, of 68 and 60 kD, were immunoprecipitated by the rabbit anti-ARSE antiserum. Both polypeptides are related to ARSE, since they were not detected in mock cells (fig. 1*a*). Short labeling times (5 min), followed by chases of 1–20 h, showed that the 60-kD polypeptide is the precursor of the enzyme. The 60-kD form was rapidly converted into the mature 68-kD polypeptide (fig. 1*b*), which corresponds to the form observed in western blot experiments. The deduced amino acid sequence of ARSE predicts a protein of 589 residues, with a putative signal peptide. The putative signal peptide consists of an N-terminal region containing positively charged residues, followed by a stretch of hydrophobic residues and a more polar C-terminal region. Ala20–Val21 and Ser23–Leu24 represent possible signal peptidase cleavage sites, since they both fulfill the ($-3$, $-1$) rule (von Heijne 1986). The molecular mass of the deduced pol-

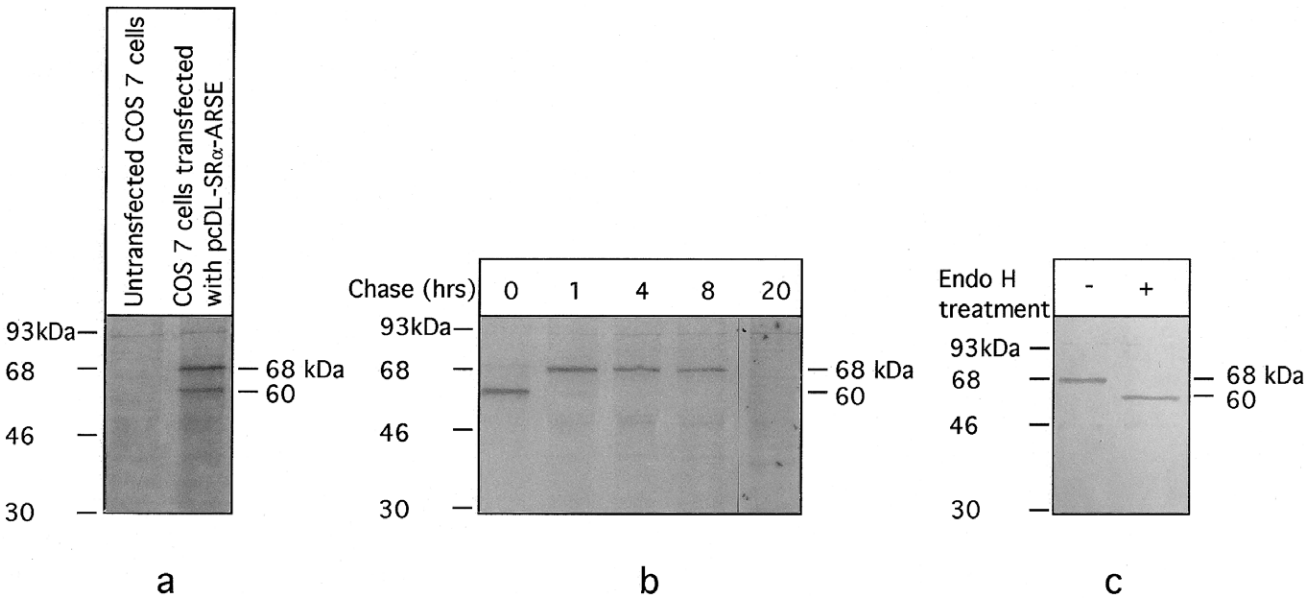


**Figure 1** *a,* Immunoprecipitation and SDS-PAGE analysis of COS cells transfected with pcDL-ARSE. Mock (*left*) and transfected (*right*) Cos7 cells were labeled with $^{35}$S-methionine for 30 min; ARSE-related polypeptides were immunoprecipitated, and the immunocomplexes were analyzed by SDS-PAGE. Two bands, of 60 and 68 kD, were detectable in the Cos7 cells transfected with the ARSE cDNA. *b,* Biosynthesis and stability of the ARSE gene product. Cos7 cells transfected with pcDL-ARSE were labeled for 5 min and were chased for variable periods. Cell extracts were immunoprecipitated, and the immunocomplexes were analyzed by SDS-PAGE. After 5 min of labeling, only the 60-kD polypeptide was detectable. This polypeptide was rapidly converted into the 68-kD form, which is the only molecular form visible after 1, 4, and 8 h of chase; after 20 h, the 68-kD polypeptide was barely detectable. *c,* Glycosylation of the ARSE gene product. Cos7 cells transfected with pcDL-ARSE were labeled for 30 min and were chased for 4 h. Cell extracts were immunoprecipitated; the immunocomplexes were denatured and subjected to endoglycosidase treatment and were analyzed by SDS-PAGE, together with the untreated immunocomplexes. After endoglycosidase H treatment, the 68-kD polypeptide was converted into the 60-kD form.

ypeptide chain, excluding the putative N-terminal signal peptide, was calculated to be ~61 kD, corresponding to the apparent SDS-PAGE molecular weight of the precursor form.

After purification of transfected Cos7 cell extract on concanavalin A-Sepharose, the band corresponding to ARSE was retained among the glycosylated proteins (fig. 4*A*). Moreover, treatment with endoglycosidase H reduced the size of the 68-kD polypeptide by 8 kD, suggesting that the increase in molecular size observed in the maturation of ARSE is due to N-glycosylation (fig. 1*c*).

### *Intracellular Localization of ARSE*

All the sulfatases identified thus far are lysosomal proteins (Stein et al. 1989*a*; Hopwood and Morris 1990; Neufeld and Muenzer 1995), with the exception of STS, which has been localized in almost all membrane compartments—the rough and smooth endoplasmic reticulum, the Golgi cisternae, the endosomes, the dense lysosomes, and the plasma membrane (Willemsen et al. 1988; Stein et al. 1989*b*). We used indirect immunofluorescence techniques to establish the subcellular localization of ARSE in transfected Cos7 cells. The anti-ARSE antibody revealed a typical Golgi-apparatus staining 12, 24, 48, and 72 h after transfection. The Golgi localization was confirmed by double staining with rhodamine-conjugated WGA, a lectin that binds with high-affinity residues of N-acetyl-D-lucosamine present in high concentration in the Golgi apparatus (Tartakoff and Vassalli 1983). As shown in figure 2*a* and *b,* the distribution of ARSE corresponds to that of WGA. The ARSE localization also was confirmed by double staining with antibodies raised against Golgi epitopes. By use of monoclonal antibodies against β-COP (clone maD) (Pepperkok et al. 1993), the immunofluorescence staining was confined mainly to the central perinuclear area in the cell, which corresponds to the results observed with anti-ARSE antiserum (fig. 2*c* and *d*), and was not superimposable with a typical ER staining revealed by parallel transfection with the ARSD cDNA (fig. 2*e* and *f*) and with STS (data not shown). A similar result was obtained by use of a monoclonal antibody against the Golgi 58K protein (clone 58K-9) (Bloom and Brashear 1989) as a marker of the Golgi apparatus (data not shown). To verify that what we found corresponds to the normal ARSE localization and not to an artifact that is due to overexpression in the Cos7 cell system, we

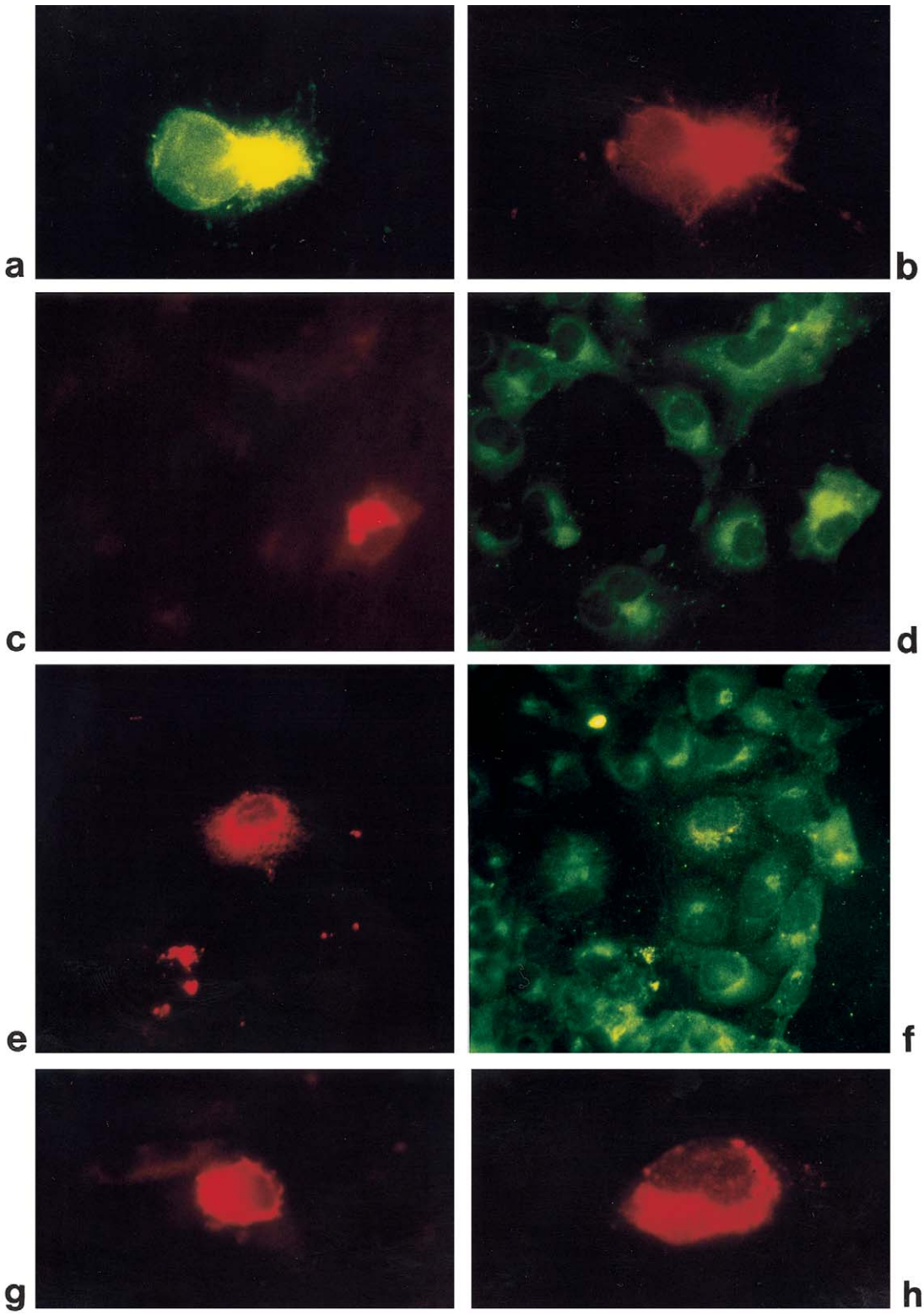


**Figure 2** Localization of ARSE in transfected cells. Cos7 cells were transfected with ARSE (*a–d*) and ARSD (*e* and *f*). NIH3T3 and RPE cells were transfected with ARSE (*g* and *h*). *a* and *b,* Double staining using anti-ARSE antiserum, followed by FITC anti-rabbit antibody, and rhodamine-conjugating WGA, respectively ( × 950). *c* and *d,* Double staining using anti-ARSE antiserum, followed by TRITC anti-rabbit antibody, and monoclonal anti-Golgi 58K protein, followed by FITC anti-mouse antibody, respectively ( × 380). *e* and *f,* Double staining using anti-ARSD antiserum, followed by TRITC anti-rabbit antibody, and monoclonal anti-Golgi 58K protein, followed by FITC anti-mouse antibody, respectively ( × 380). *g* and *h,* Single staining using anti-ARSE antiserum, followed by TRITC anti-rabbit antibody ( × 950).

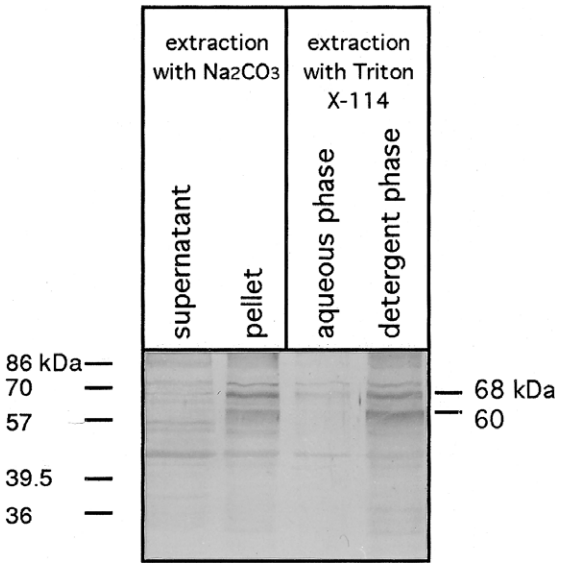


**Figure 3** Immunoblot of ARSE-transfected Cos7 cells solubilized with either $Na_2CO_3$ (lanes 1 and 2) or Triton X-114 (lanes 3 and 4). Lane 1, Supernatant after extraction with $Na_2CO_3$. Lane 2, Pellet. After extraction with Triton X-114, the extract was separated into the aqueous (lane 3) and detergent (lane 4) phases.

tested several cell lines expressing a lower level of transfected ARSE protein. Figure 2*g* and *h* shows localization of this protein to the Golgi apparatus, in two of these cell lines, NIH3T3 and RPEmsma (Schiaffino et al. 1996).

ARSE could not be solubilized by 0.5 M $Na_2CO_3$, whereas, after Triton X-114 extraction, ARSE protein was recovered in the detergent phase (fig. 3). This result suggests that ARSE behaves as an integral membrane protein. Similar to STS, ARSE contains two putative membrane-spanning domains (amino acids 199–216 and amino acids 224–244) that are not present in ARSA and ARSB, as was deduced from the hydrophobicity profile (Peters et al. 1990; Franco et al. 1995). These domains could be responsible for the anchoring of ARSE to the Golgi cisternae membranes.

### *Arylsulfatase Activity of ARSE Mutants*

Single nucleotide changes that produce the amino acid substitutions R12S, R111P, G137V, G245R, and C492Y were identified in patients affected with CDPX (Franco et al. 1995; Parenti et al., 1997). To investigate the effect of these missense mutations, the nucleotide changes leading to these amino acid substitutions were introduced into the wild-type ARSE expression vector by site-directed mutagenesis. Wild-type and mutant expression plasmids were transiently transfected into Cos7 cells by electroporation. After 48–72 h, the total cell extracts and the culture medium were assayed for their ability to hydrolyze the sulfate group from 4MU sulfate. No residual arylsulfatase activity was detectable in the culture medium of the cells transfected with the wild-type and mutant constructs. The extracts from cells transfected with R111P, G137V, G245R, and C492Y mutant constructs exhibited significantly lower arylsulfatase activity, with loss of the catalytic properties of the ARSE enzyme. The R12S construct exhibited activity comparable to that of the normal enzyme (table 1). We studied the effect of this latter mutation in relation to the pH optimum and thermostability of the enzyme and on the activity in the presence of Warfarin as an inhibitor

**Table 1**

**Arylsulfatase Activity of ARSE Mutants Transiently Transfected in Cos7 Cells**

| | Arylsulfatase Activity (nmol 4MU/mg/h) | | | | | | | | | Average Normalized Arylsulfatase Activity[b] (nmol 4MU/mg/h) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Experiment 1 | | | Experiment 2 | | | Experiment 3 | | | |
| Cell Type | Raw Data | Luciferase Units | Normalized[a] | Raw Data | Luciferase Units | Normalized[a] | Raw Data | Luciferase Units | Normalized[a] | |
| Wild type | 28.5 | 13,801 | 28.5 | 20 | 58,151 | 20 | 10.98 | 13,3728 | 10.98 | 15.3 ± 3.9 |
| R12S | 40 | 18,870 | 29.25 | 24 | 72,617 | 19.21 | 14.38 | 14,9721 | 12.84 | 20.40 ± 4.7 |
| R111P | .16 | 8,492 | .26 | .31 | 94,878 | .19 | .35 | 187,219 | .25 | .26 ± .05 |
| G137V | 1.5 | 31,091 | .66 | .7 | 107,003 | .38 | .43 | 113,862 | .5 | .51 ± .08 |
| G245R | .41 | 16,640 | .34 | .27 | 98,129 | .16 | .71 | 186,170 | .51 | .33 ± .1 |
| C492Y | .85 | 13,010 | .9 | .09 | 76,892 | .068 | .11 | 173,666 | .08 | .34 ± .27 |
| Cos7 | .32 | | | .14 | | | .23 | | | .23 ± .09 |

Note.—To control transfection efficiency and variability, all results were normalized by testing luciferase activity after cotransfection with a luciferase reporter gene vector.

[a] By comparison of the luciferase activity of the mutants with the luciferase activity of the wild-type construct, to correct for transfection variability.

[b] Average of the normalized activity results of at least four independent experiments.

(Franco et al. 1995). The R12S mutation behaved like the wild-type enzyme, with respect to all these parameters (data not shown).

### *Detection of Mutated ARSE Protein Product*

The presence of mutated ARSE polypeptide in transfected Cos7 cell extracts was determined by immunoblot analysis using anti-ARSE antibody. Although no enzyme activity was expressed from the transfected cDNA, the amount of ARSE crossreacting material visualized by western blot was comparable to that found in the cells expressing the wild-type cDNA. As expected, the amount of crossreacting material of R12S also was comparable to that found using the wild-type cDNA (fig. 4*A*). To test for decreased stability of the mutant proteins, Cos7 cells transfected with the ARSE cDNA carrying different point mutations were labeled for 30 min and were chased for 1 , 4, 8, and 20 h. All ARSE mutants showed apparently normal processing and stability. The biosynthesis of the ARSE gene product carrying the substitution G137V is shown in figure 4*B*. Therefore, four of these mutations cause a severe reduction in the activity of the enzyme without altering the production and stability of the protein.

Since all ARSE mutants produced stable proteins, we investigated the possibility that these amino acid substitutions could lead to a mislocalization of the enzyme. Particularly, the R12S mutation residing in the putative signal peptide and exhibiting normal arylsulfatase activity was a candidate for this kind of alteration. Indirect immunofluorescence data did not show any difference in the distribution of this mutant protein (fig. 4*C*) or of the other mutants (data not shown), with respect to the wild-type protein. All these amino acid substitutions, with the exception of R12S, lead to protein alterations affecting the catalytic properties of ARSE, which proves their involvement in the pathogenesis of CDPX.

## Discussion

Although mutations in the ARSE gene cause CDPX, the pathogenesis of the disease and the physiological role of ARSE are still unknown. A number of observations suggest that sulfate metabolism is essential for the correct composition of the bone and cartilage matrix (Hästbacka et al. 1994; Superti-Furga 1994). To try to better understand the physiological role of this new arylsulfatase, we started a biochemical characterization of the wild-type ARSE and of the mutants found in CDPX patients.

We detected two molecular forms of ARSE, a 60-kD and a 68-kD protein, in cell extracts after transfection. The 60-kD polypeptide is quickly converted into a stable 68-kD form, which is likely to represent the mature enzyme, with an increase in size due to N-glycosylation. The amino acid sequence contains four potential N-glycosylation sites (asparagine residues 58, 125, 258, and 344). Assuming that the average mass of an oligosaccharide is 2 kD, all four N-glycosylation sites possibly are utilized, accounting for the 8-kD shift observed in SDS-PAGE. The STS predicted sequence shows four potential N-glycosylation sites, of which two are utilized (Stein et al. 1989*b*). Interestingly, alignment of all human sulfatases (Franco et al. 1995) indicates that three of four STS potential N-glycosylation sites (Asn 47, 259, and 333) are present in the ARSE sequence (Asn 58, 258, and 344), in the corresponding positions.

Localization of ARSE exclusively in the Golgi apparatus is a unique feature among the sulfatases. Most human sulfatase enzymes that have been identified are localized in the lysosomes (Stein et al. 1989*a*; Hopwood and Morris 1990; Neufeld and Muenzer 1995). The only exception is STS, which is distributed in all the cellular membrane compartments (rough and smooth endoplasmic reticulum, the Golgi apparatus, and lysosomes) and on the plasma membrane (Willemsen et al. 1988; Stein et al. 1989*b*). ARSE is likely to be anchored to the Golgi cisternae, through the two predicted transmembrane domains, since it is not solubilized by $Na_2CO_3$ or 0.5 M KCl (data not shown) and since it behaves as an integral membrane protein, after extraction with Triton X-114. The distribution of the enzyme in a neutral pH compartment is consistent with the pH optimum observed for ARSE arylsulfatase activity (Franco et al. 1995). More detailed experiments are required to define the exact distribution of ARSE within the Golgi subcompartments and to determine its topology in the membrane, although these preliminary data on its localization may help in the identification of the natural substrate.

These biochemical findings, together with previous data, indicate that the sulfatase gene cluster in Xp22.3 (ARSD, ARSE, ARSF, and STS) represents a subfamily of arylsulfatases of relatively recent origin. In addition to sharing the highest degree of sequence homology (Franco et al. 1995) and the same genomic organization (Meroni et al. 1996), these sulfatases show a putative transmembrane domain that is absent in ARSA and ARSB. Immunofluorescence results indicate that ARSD and ARSF (G. Meroni, unpublished data) are ER proteins, confirming the nonlysosomal nature of the Xp22.3 sulfatases.

Evidence that ARSE is implicated in CDPX was derived from indirect observations. Screening by SSCP and sequence analysis of DNA from 27 unrelated patients with phenotypes comparable to that of CDPX revealed point mutations in six of these patients, in the coding region of the ARSE gene; these mutations were not found in 120 control chromosomes (Franco et al. 1995; Parenti

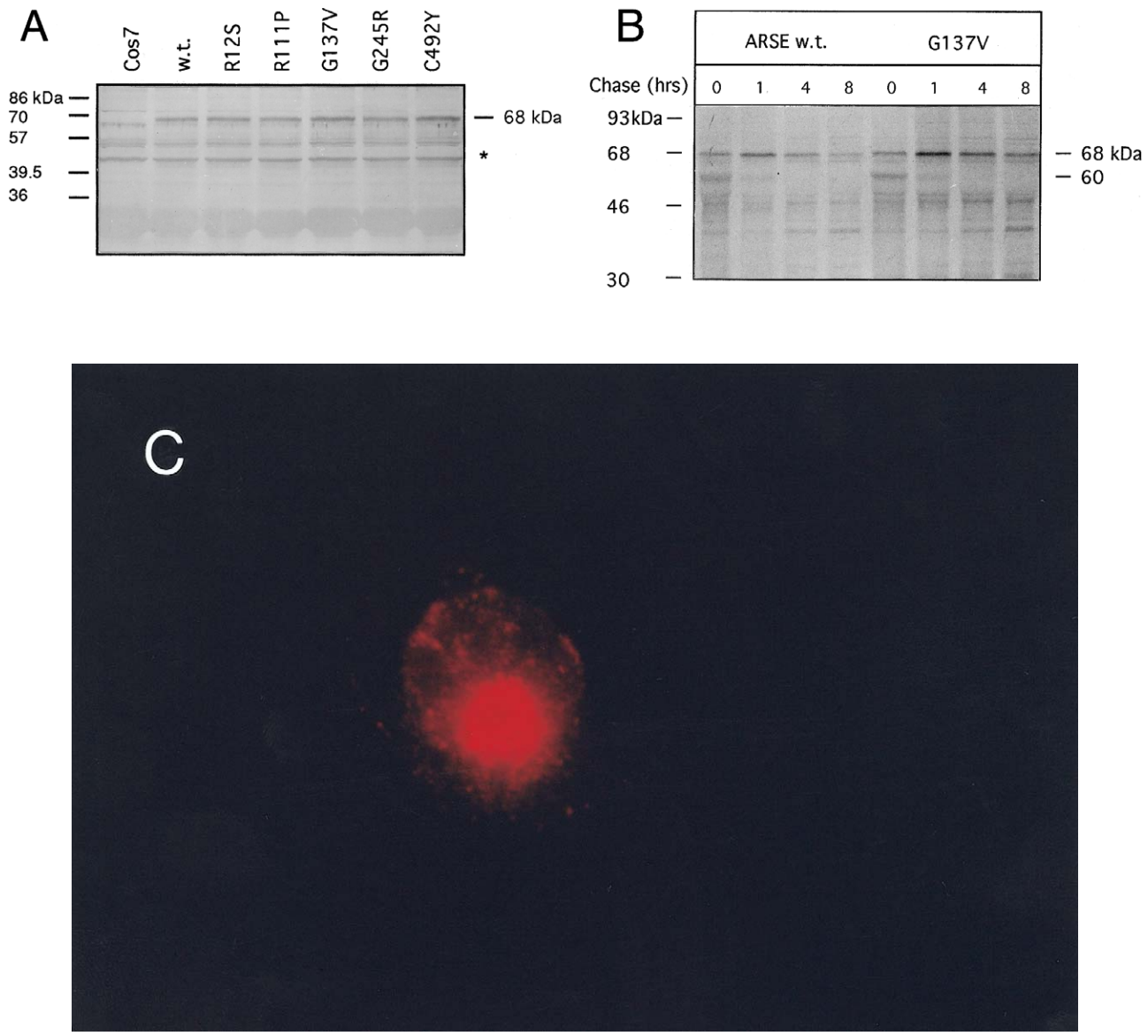


**Figure 4** *A*, Immunoblot analysis of ARSE mutants. Total extracts of cells transfected with the ARSE wild-type and mutant cDNAs were subjected to SDS-PAGE, followed by immunoblotting with anti-ARSE antiserum. *B*, Biosynthesis and stability of the wild-type and G137V mutant ARSE. Cos7 cells transfected with the constructs carrying either the wild-type or the mutated ARSE cDNA were labeled for 30 min and were chased for variable periods. Cell extracts were immunoprecipitated, and the immunocomplexes were subjected to SDS-PAGE analysis; the results obtained with the construct carrying the mutant ARSE cDNA are shown (*right*) as compared with the results obtained with the wild-type cDNA (*left*). *C*, R12S ARSE-transfected Cos7 cells stained with anti-ARSE antiserum, followed by TRITC anti-rabbit antibody.

et al., 1997). Other nucleotide substitutions leading to missense mutations have been found in the ARSE coding region (L. Sheffield, personal communication). In contrast, no mutations were found in the ARSD and ARSF genes. Genetic heterogeneity in patients with phenotypes comparable to that of CDPX has been suggested (Franco et al. 1995), to explain ARSE mutations that have been found only in a minority of the screened patients. However, formal proof that the mutations in the ARSE sequence interfere with the activity of the ARSE gene product was not available. The mutations were scattered over the entire sequence of the cDNA, involving variably conserved amino acid residues of the ARSE protein. Two of the mutations resulted in nonconservative amino acid substitutions.

Many amino acid substitutions in the known sulfatases have proved to be responsible for the deficiency of activity observed in the respective disorders. In recent years, much attention has been paid to the mechanisms involved in the hydrolysis of sulfate ester bonds by sul-

fatases. A major breakthrough in the understanding of the function of sulfatases has emerged recently from the observation that sulfatases undergo a common and unique co- or posttranslational modification, probably occurring in the endoplasmic reticulum, in which a cysteine residue is converted into a 2-amino-3-oxopropionic acid or a serine semialdehyde. Moreover, this finding has been fundamental to uncovering the basis of the MSD disorder (Schmidt et al. 1995; Selmer et al. 1996). Recently, the study of the crystal structure of ARSB (Bond et al. 1997) has added additional insight into the mechanisms of sulfate hydrolysis by sulfatases. The active site of the enzyme has been characterized and has been shown to display unique features. Modified cysteine residue 91 and a metal ion are located at the base of a substrate-binding pocket. Some amino acid residues conserved throughout the sulfatase family play a role in stabilizing the calcium ion and the sulfate ester in the active site.

To confirm the correlation between the mutations and the occurrence of CDPX, we reproduced in vitro, by site-directed mutagenesis, five of the mutations described previously. The ARSE full-length cDNAs carrying the mutations were expressed in Cos cells, and the effects of the mutations on activity, biosynthesis, and subcellular localization of the enzyme were studied. Since the mutated constructs yielded a relatively normal level of ARSE polypeptide with regular molecular size, stability, and subcellular distribution, they probably do not induce dramatic structural changes in the protein.

Four of the mutated constructs, however, directed the synthesis of polypeptides with severely reduced activity, indicating that the mutated amino acids are critical for the catalytic activity of the ARSE enzyme. The nucleotide alteration leading to the R12S substitution did not appeared to alter the enzyme activity, compared with the wild-type ARSE construct. Immunofluorescence studies have shown that COS cells transfected with R12S cDNA contain a normal level of the ARSE polypeptides in the Golgi compartment, ruling out the hypothesis that this mutation, which is located in the putative leader sequence, could affect the targeting of the ARSE protein to the correct subcellular compartment. A possible explanation is that the R12S is in fact a rare neutral mutation. In principle, the hypothesis that the R12S ARSE enzyme retains activity toward the artificial substrate 4MU sulfate but is catalytically inactive against its natural substrate cannot be excluded. The mutation that changes a glycine residue at position 137 to a valine was considered to be of particular interest. In fact, glycine 137 is located in a highly conserved region and is found at the same position in all known human sulfatases. Furthermore, a mutation involving the glycine residue at the same position in the ARSB gene already has been detected in a patient with mucopolysaccharidosis VI (Maroteaux-Lamy syndrome; Wicker et al. 1991). The authors demonstrated that this mutation severely impaired the processing and stability of the ARSB protein, whereas the catalytic activity of the enzyme was not affected. Our findings showed that the G137V ARSE protein is synthesized normally but that the catalytic activity, tested by use of the artificial substrate 4MU sulfate, is reduced markedly. Therefore, although this residue is conserved between the two arylsulfatases, the same alteration probably leads to different consequences for the enzyme's properties, likely owing to the different processing and topology of the two sulfatases in the cell.

The mutations R111P and G245R, in exons 4 and 5, respectively, resulted in two nonconservative amino acid substitutions, changing a basic amino acid to a nonpolar amino acid and vice versa. It should be noted that the substitution of an arginine 111 with a proline residue could interrupt the correct secondary structure of the ARSE enzyme, introducing an abnormal turn in a conserved region of the protein. Mutation C492Y, in exon 10, involves a residue that is conserved only among the Xp22.3 sulfatases. This substitution may interfere with the formation of a disulfide bond.

Alignment of the ARSE amino acid sequence with those of all the other known sulfatases revealed that all but one of the residues (His in position 354 of ARSE, which is Ash in position 301 of ARSB) involved in metal ion coordination and stabilization of the sulfate ester are conserved, on the basis of the crystal structure of ARSB (Bond et al. 1997). The mutations found in CDPX patients are scattered along the entire length of the protein, but none of these mutations involve the amino acids that form the catalytic pocket. These mutations do not affect the active site of the enzyme and still allow the production of an apparently correct protein, in terms of stability and processing. Therefore, until new data are available, we can theorize only that these substitutions are essential for the fine folding required for the production of a catalytically active enzyme.

In conclusion, we have confirmed that four of the mutations identified in CDPX patients are very likely to be the cause of the disease. Although the sulfatases share regions of high sequence similarity along their entire lengths, little is known about the structure-function relationship of these sequences. The newly defined crystal structure, together with single- or multiresidue mutagenesis, will help to define the sequence-function relationship in this family of enzymes.

## Acknowledgments

We are grateful to Rosaria Tuzzi for technical work. We thank Elena I. Rugarli and Brunella Franco for critically reading the manuscript and Melissa Smith for help in preparing the manuscript. The support of the Italian Téléthon Founda-

tion is gratefully acknowledged. This work also was supported by Téléthon grant E.283 (to G.P.).

## References

Ballabio A, Parenti G, Carrozzo R, Sebastio G, Andria G, Buckle V, Fraser N, et al (1987) Isolation and characterization of a steroid sulphatase cDNA clone: genomic deletions in patients with X-chromosome-linked ichthyosis. Proc Natl Acad Sci USA 84:4519–4523

Ballabio A, Shapiro LJ (1995) STS deficiency and X-linked ichthyosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 2999–3022

Bloom GS, Brashear TA (1989) A novel 58-kDa protein associates with the Golgi apparatus and microtubules. J Biol Chem 264:16083–16092

Bond CS, Clements PR, Ashby SJ, Collyer CA, Harrop SJ, Hopwood JJ, Guss JM (1997) Structure of a human lysosomal sulfatase. Structure 5:277–289

Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 256:1604–1607

Curry CJR, Magenis E, Brown M, Lanman JT Jr, Tsai J, O'Lague P, Goodfellow P, et al (1984) Inherited chondrodysplasia punctata due to a deletion of the terminal short arm of an X chromosome. N Engl J Med 311:1010–1014

Franco B, Meroni G, Parenti G, Levilliers J, Bernard L, Gebbia M, Cox L, et al (1995) A cluster of sulfatase genes on Xp22.3: mutations in chondrodysplasia punctata (CDPX) and implications for Warfarin embryopathy. Cell 81:15–25

Fujiki Y, Hubbard AL, Fowler S, Lazaro PB (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. J Cell Biol 93:97–102

Hall JG, Pauli RM, Wilson KM (1980) Maternal and fetal sequelae of anticoagulation during pregnancy. Am J Med 68:122–140

Hästbacka J, de la Chapelle A, Mahtani MM, Clines G, Reeve-Daly MP, Daly M, Hamilton BA, et al (1994) The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. Cell 78:1073–1087

Hopwood JJ, Morris CP (1990) The mucopolysaccharidoses: diagnosis, molecular genetics and treatment. Mol Biol Med 7:381–404

Kolodny EH, Fluharty AL (1995) Metachromatic leukodystrophy and multiple sulfatase deficiency: sulfatide lipidosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 2693–2741

Meroni G, Franco B, Archidiacono N, Messali S, Andolfi G, Rocchi M, Ballabio A (1996) Characterization of a cluster of sulfatase genes on Xp22.3 suggests gene duplications in an ancestral pseudoautosomal region. Hum Mol Genet 5: 423–431

Neufeld EF, Muenzer J (1995) The mucopolysaccharidoses. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 2465–2494

Nordeen SK (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques 6:454–457

Parenti G, Buttitta P, Meroni G, Franco B, Bernard L, Rizzolo MG, Brunetti-Pierri N, et al. (1997) X-linked recessive chondrodysplasia punctata due to a new point mutation of the ARSE gene. Am J Med Genet 73:139–143

Parenti G, Meroni G, Ballabio A (1997) The sulfatase gene family. Curr Opin Genet Dev 7:386–391

Pauli RM, Lian JB, Mosher DF, Suttie JW (1987) Association of congenital deficiency of multiple vitamin K–dependent coagulation factors and the phenotype of the Warfarin embryopathy: clues to the mechanism of teratogenicity of coumarin derivatives. Am J Hum Genet 41:566–583

Pepperkok R, Scheel J, Horstmann H, Hauri HP, Griffiths G, Kreis TE (1993) $\beta$-cop is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. Cell 74:71–82

Peters C, Schmidt B, Rommerskirch W, Rupp K, Zühlsdorf M, Vingron M, Meyer HE, et al (1990) Phylogenetic conservation of arylsulfatases cDNA cloning and expression of human arylsulfatase. J Biol Chem 265:3374–3381

Puca A, Zollo M, Repetto M, Andolfi G, Guffanti A, Simon G, Ballabio A, et al (1997) Identification by shotgun sequencing, genomic organization, and functional analysis of a fourth arylsulphatase gene (ARSF) from the Xp22.3 region. Genomics 42:192–199

Schiaffino MV, Baschirotto C, Pellegrini G, Montalti S, Tacchetti C, De Luca M, Ballabio A (1996) The ocular albinism type I (OA1) gene product is a membrane glycoprotein localized to melanosomes. Proc Natl Acad Sci USA 93: 9055–9060

Schmidt B, Selmer T, Ingendoh A, yon Figura K (1995) A novel amino acid modification in sulfatases that is defective in multiple sulfatase deficiency. Cell 82:271–278

Selmer T, Hallmann A, Schmidt B, Sumper M, Von Figura K (1996) The evolutionary conservation of a novel protein modification, the conversion of cysteine to serine semialdehyde in arylsulfatase from *Volvox carteri*. Eur J Biochem 238:341–345

Stein C, Gieselmann V, Kreysing J, Schmidt B, Pohlmann R, Waheed A, Meyer HE, et al (1989*a*) Cloning and expression of human arylsulfatase A. J Biol Chem 264:1252–1259

Stein C, Hille A, Seidel J, Rijnbout S, Waheed A, Schmidt B, Geuze H, et al (1989*b*) Cloning and expression of human steroid-sulfatase. J Biol Chem 264:13865–13872

Superti-Furga A (1994) A defect in the metabolic activation of sulfate in a patient with achondrogenesis type IB. Am J Hum Genet 55:1137–1145

Takebe Y, Seiki M, Fujisawa J-I, Hoy P, Yokota K, Arai K-I, Yoshida M, et al (1988) SR$\alpha$ promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol Cell Biol 8:466–472

Tartakoff AM, Vassalli P (1983) Lectin-binding sites as markers of Golgi subcompartments: proximal-to-distal maturation of oligosaccharides. J Cell Biol 97:1243–1248

von Heijne G (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 14:4683–4690

Wang I, Franco B, Ferrero GB, Chinault AC, Weissenbach J,

Chumakov I, Le Paslier D, et al (1995) High-density physical mapping of a 3-Mb region in Xp22.3 and refined localization of the gene for X-linked recessive chondrodysplasia punctata (*CDPX1*). Genomics 26:229–238

Wicker G, Prill V, Brooks D, Gibson G, Hopwood J, von Figura K, Peters C (1991) Mucopolysaccharidosis VI (Maroteaux-Lamy syndrome). J Biol Chem 266:21386–21391

Willemsen R, Kroos M, Hoogeveen AT, van Dongen JM, Parenti G, van der Loos CM, Reuser AJJ (1988) Ultrastructural localization of steroid sulphatase in cultured human fibroblasts by immunocytochemistry: a comparative study with lysosomal enzymes and the mannose 6-phosphate receptor. Histochem J 20:41–51

Yen PH, Allen E, Marsh B, Mohandas T, Wang N, Taggart RT, Shapiro LJ (1987) Cloning and expression of steroid sulfatase cDNA and the frequent occurrence of deletions in STS deficiency: implication for X-Y interchange. Cell 49: 443–454